

Short Communication

Epstein–Barr Virus in Gastric Carcinoma

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Epstein–Barr virus (EBV) is known to be related to lymphoid tumors and some types of epithelial tumors, including lymphoepithelioma-like gastric carcinoma with marked lymphocytic stroma. In this study, prevalence of EBV involvement in gastric cancer, and characteristics of tumors with such involvement, were investigated by EBV-encoded RNA 1 in situ hybridization applied to paraffin sections, including the tumor and adjacent gastric tissue, from 999 gastric carcinomas observed in 970 consecutive cases from a large Japanese hospital. EBV involvement occurred in 6.9 percent of lesions, a significantly lower proportion than has been observed in a North American series. Involvement was significantly more frequent among males, in tumors in the upper part of the stomach, and in adenocarcinomas of the moderately differentiated tubular and poorly differentiated solid or medullary types. Almost all carcinomas with marked lymphoid stroma were EBV-positive. Positive lesions were characterized by the presence of uniform hybridized signals in almost all carcinoma cells and by their absence from adjacent non-neoplastic tissue. (Am J Pathol 1993, 143:1250–1254)

Involvement of Epstein–Barr virus (EBV) DNA has been reported in tumor cells of gastric carcinoma with intense lymphocytic infiltration, a type that has histo-

logical resemblance to nasopharyngeal lymphoepithelioma.^{1–4} Also, Shibata and Weiss reported finding EBV in 16% of a series of U.S. cases with typical gastric adenocarcinoma, using polymerase chain reaction and *in situ* hybridization (ISH).⁵

Elsewhere (Imai S, and Tokunaga M, in preparation), we report detecting monoclonality of EBV terminal repeats by Southern blot analysis using five EBV-positive gastric carcinomas. Serum antibody titers of EBV-positive cases showed markedly elevated levels of immunoglobulin G antibodies against viral capsid antigen and early antigen and increased positive rates of immunoglobulin A antibodies against viral capsid antigen and early antigen.

ISH using anti-sense probe for EBV-encoded small RNA (EBER) is a highly sensitive method for detecting EBV-infected cells.^{6,7} The aim of the present study was to determine the frequency of EBV-related gastric carcinoma in Japanese cases using EBER-1 ISH and to clarify the clinicopathological characteristics of such tumors.

Materials and Methods

Formalin-fixed, paraffin-embedded blocks from 999 gastric carcinomas observed in 970 cases were studied. There were 26 cases with multiple gastric carcinomas; 24 were double carcinomas, one a triple, and one quadruple. The source was consecutive gastrectomy cases listed in the Pathology Department of Kagoshima City Hospital between 1976 and August 1992. Paraffin blocks taken from the main tumors were used for hematoxylin and eosin staining and ISH. The gastric carcinomas were principally classified as intestinal and diffuse by the criteria of Lauren.⁸ These two types of carcinomas

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were subclassified by predominant histological pattern, using the classification scheme of the Japanese research society for gastric cancer⁹ and its revision as follows: intestinal types tub1 (well-differentiated tubular adenocarcinoma with tubule formation of carcinoma cells in almost all areas) and tub2 (moderately differentiated tubular adenocarcinoma); diffuse types por1 (solid tumor type with a medullary pattern of poorly differentiated adenocarcinoma), por2 (nonsolid tumor type with diffuse proliferation with or without scirrhous pattern), sig (signet ring cell carcinoma), and muc (mucinous carcinoma). We also specified a special category LE (lymphoepithelioma-like carcinoma with intensive lymphoid stroma, generally poorly differentiated adenocarcinoma or undifferentiated carcinoma, rarely with tubular structures, in which the amount of lymphoid stroma usually exceeds total mass of the carcinoma cells). Ten cases with malignant lymphoma and two leiomyomas were not included in the study; all were negative for EBER-1 ISH. Site of tumor was classified according to the predominant location of the tumor as cardia, middle portion, and antrum. Lesions occurring in the gastric stump, for cases previously gastrectomized for peptic ulcer, were considered separately. The depth of invasion was classified as follows: mucosa, submucosa, propria muscular, or subserosa.

In Situ Hybridization

The EBV RNA sequence EBER-1 was detected with a complementary digoxigenin-labeled 30-base oligomer, using a procedure described elsewhere (Chang KL, Chen Y-Y, Shibata D, et al., in preparation). Five- μ paraffin sections cut from the main tumor were deparaffinized, rehydrated, predigested with pronase, prehybridized, and hybridized overnight at 37 C with a concentration of 0.5 ng of digoxigenin-labeled probe. After washing by 0.5 \times standard saline citrate, hybridization was detected by an antidigoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannheim, Mannheim, Germany) as suggested by the manufacturer. Lymph node sections from a patient with infectious mononucleosis were used for positive control, and a sense probe for EBER-1 was used for negative control for each procedure.

Statistical evidence regarding differences in EBV prevalence by age, year, sex, site, histological type, and invasiveness was obtained by linear logistic regression using the general models for binary out-

comes algorithm from the EPICURE package of statistical programs for analysis of epidemiological data.¹⁰

Results

Sixty-seven cases (6.9%) and 69 carcinomas (6.9%) were EBV-positive, a rate significantly lower ($P = 0.0005$) than the 16% observed in a North American series by Shibata and Weiss.⁵ EBER-1 ISH signals were observed uniformly in the nuclei and nucleoli, but cytoplasm was negative (Figure 1a). Adjacent non-neoplastic gastric mucosa with chronic gastritis, atrophic gastritis, or intestinal metaplasia were completely negative. Lesions of adenoma and hyperplastic polyp were also negative. There were 10 instances in which EBER-1 ISH signals were seen in the lymphocytes around the carcinoma, but almost all lymphocytes observed in the mucosa were EBV-negative. Two cases with double carcinomas were positive in both, and another two double cases were positive in one and negative in the other.

Age, Sex, and Year of Diagnosis

There was no significant variation in EBV prevalence by age (21 to 88) or calendar year of diagnosis. EBV involvement was significantly more prevalent among males (56 cases, or 9.2%) than females (11 cases, 3.1%), corresponding to an unadjusted odds ratio of 3.3 for males versus females (95% confidence limits 1.8 and 6.7, $P < 0.0001$).

Tumor Site

There was significant variation in EBV prevalence by site (Table 1). Over half of the tumors were located in the antrum, and of these only 20 (3.9%) were EBV-positive. Positive findings were relatively more frequent in the cardia (8.1%), middle stomach (10.6%), and in the gastric stump (12.5%).

Histological Type

EBV involvement was observed in papillary as well as tubular adenocarcinomas (Figure 1a). The division of tumors into intestinal and diffuse types was unrelated to EBV prevalence (Table 2), but within the two classes there was significant nonhomogeneity by type. Of the intestinal types, EBV involvement was significantly greater among moderately differentiated (tub2) (Figure 1b) as compared to well-differentiated (tub1) adenocarcinomas (9.1%

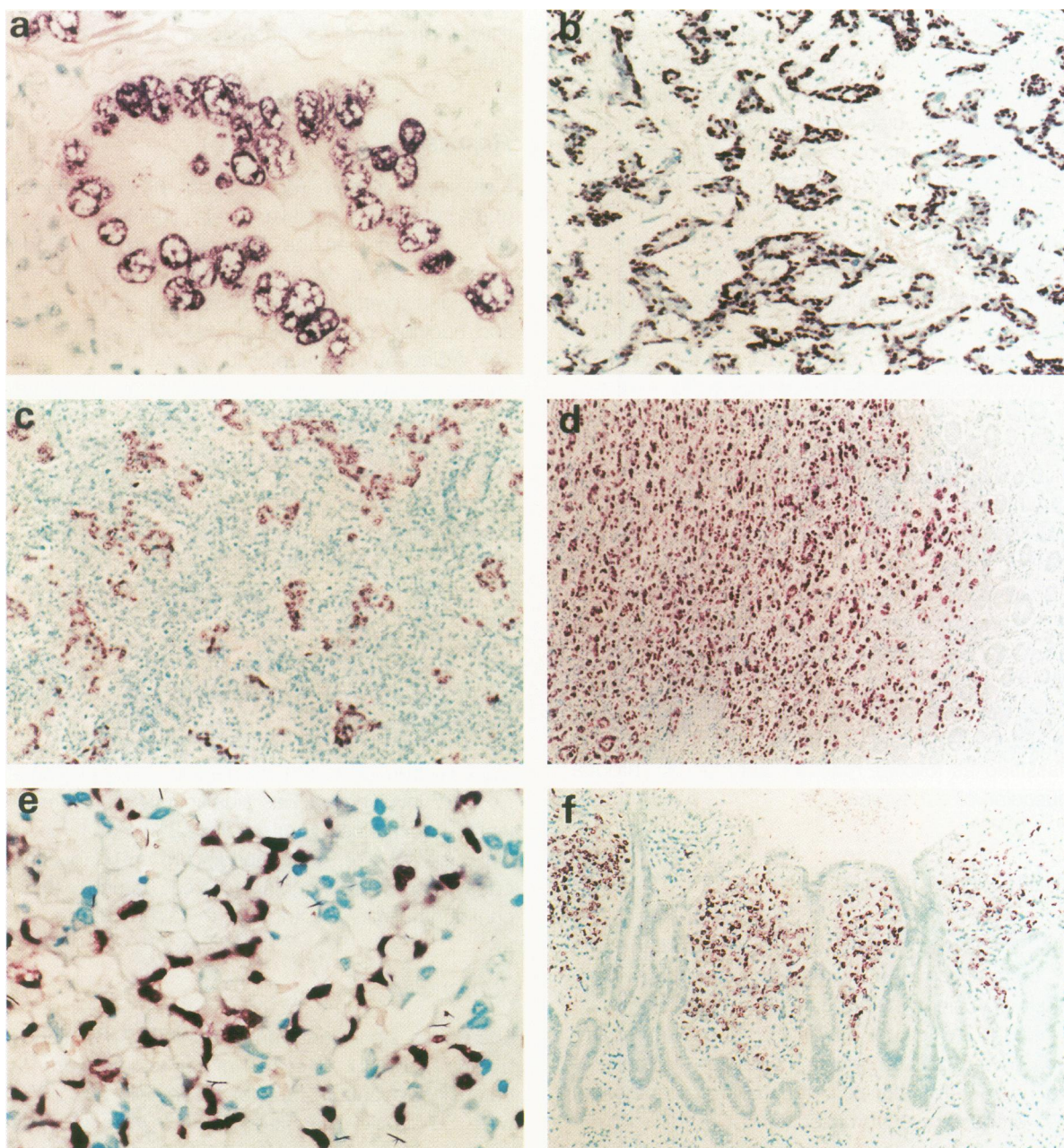


Figure 1. Purple-black hybridized signals by EBER-1 ISH, restricted in the nuclei and nucleoli of carcinoma cells of well-differentiated tubular adenocarcinoma (a), moderately differentiated tubular adenocarcinoma (b), carcinoma with lymphoid stroma (c), solid type, poorly differentiated adenocarcinoma (d), signet ring cell carcinoma (e), and intramucosal carcinoma without reaction in the adjacent mucosal epithelia (f).

vs 3.9%, $P = 0.018$). Eight of the nine LEs (Figure 1c), were EBV-positive as compared to 5.5% of other diffuse tumors. In the latter group, however, there was also significant nonhomogeneity by type ($P = 0.036$). Nineteen (8.8%) instances of EBV involvement were seen among 217 por1 tumors (Figure 1d); positive tumors showed slight to moderate lymphocytic infiltration in and around the tumor nests. EBV involvement was rarely observed in the por2, sig (Figure 1e), or muc carcinomas.

Depth of Invasion

EBV-positive carcinomas occurred at all depths of invasion, at about the same relative frequencies: six (4.4%) of 137 located in the mucosa, 16 (10.0%) of 160 in the submucosa, eight (6.9%) of 116 in the propria muscular, and 39 (6.7%) of 586 with invasion of the subserosa. EBV-positive early carcinomas were observed of both the intramucosal diffuse (Figure 1f) and intestinal types.

Table 1. *Frequency of EBV-Positive Carcinomas by Sex and Site*

	Total		Females		Males	
	Positive/lesions	%	Positive/lesions	%	Positive/lesions	%
Total	66/999	(6.9)	11/369	(3.0)	58/630	(9.2)
Cardia	11/135	(8.1)	1/32	(3.1)	10/103	(9.7)
Middle	36/341	(10.6)	1/138	(0.7)	35/203	(17.2)
Antrum	20/507	(3.9)	7/192	(3.6)	13/315	(4.1)
Gastric stump	2/16	(12.5)	2/7	(28.6)	0/9	(0.0)

Tests of homogeneity (*P* value): sex: 0.0001; site: 0.0017; site excluding gastric stump: 0.0007.

Table 2. *Frequency of EBV-Positive Carcinomas by Sex and Histological Type (A) and Tests of Homogeneity by Histological type: P values (B)*

	Total		Females		Males	
	Positive/lesions	%	Positive/lesions	%	Positive/lesions	%
Total	69/999	(6.9)	11/369	(3.0)	58/630	(9.2)
Intestinal	35/514	(6.8)	6/241	(2.5)	28/244	(11.5)
tub1	9/228	(3.9)	2/50	(4.0)	7/178	(3.9)
tub2	26/286	(9.1)	3/78	(3.8)	23/208	(11.1)
Diffuse	34/485	(7.0)	5/128	(3.9)	30/386	(7.8)
por1	19/217	(8.8)	2/106	(1.9)	17/111	(15.3)
por2	4/170	(2.4)	0/86	(0.0)	4/84	(4.8)
sig	2/52	(3.8)	1/29	(3.4)	1/23	(4.3)
muc	1/37	(2.7)	1/17	(5.9)	0/20	(0.0)
LE	8/9	(88.9)	2/3	(66.7)	6/6	(100.0)
	All types		Intestinal vs. diffuse		Intestinal subtypes	Diffuse subtypes
All types in class	<0.0001		0.90		0.018	<0.0001
All types except LE	0.010		0.38		—	0.036

Discussion

EBV is associated with a variety of malignant lymphomas, including Burkitt's lymphoma, Hodgkin's disease, B-cell lymphoma in immunodeficient individuals, and T-cell lymphomas.^{6,11-14} Following the initial serological demonstration of EBV in relation to nasopharyngeal lymphoepithelioma, EBV DNA has been detected in the tumor cells of LE-like carcinomas in several organs.^{12,14-18} Recently, EBV-related carcinomas have also been observed in some carcinomas without lymphocytic reaction.^{5,15}

Gastric carcinoma with lymphoid stroma has been reported mainly in Asian patients.^{1,2,19-22} The infiltrated lymphocytes are exclusively T cells with close attachment to the tumor cells.^{20,23} EBV involvement is seen in almost all of these LE-like carcinomas according to results presented here and in our previous study.¹

The criterion for EBV-related gastric carcinoma in this study is the demonstration of EBER-1 by ISH in almost all tumor cells. Because of the uniform specific distribution of ISH signals of EBER-1 in the tumor cells within a given positive case, there were no positive cancers with sporadic focal distribution of

EBER-1 ISH in the tumor cells. These findings suggest that EBV infection occurs at an early stage in carcinogenesis with a subsequent monoclonal expansion of EBV-containing tumor cells^{5,24} (Imai S, and Tokunaga M, in preparation).

Although Shibata and Weiss reported EBV genomes in the dysplastic epithelium adjacent to carcinoma,⁵ we could not detect EBER-1 ISH in any adenoma, hyperplastic polyp, intestinal metaplasia, or atrophic gastritis, or in the normal gastric mucosal epithelium. All epithelial cells found to be EBV-positive by EBER-1 ISH seemed to be carcinoma cells, even in well-differentiated papillary lesions. These findings and the lack of expression of CD21 observed by us in non-neoplastic gastric mucosa and in carcinoma cells, using frozen materials, suggests the existence of an entrance mechanism for EBV into gastric epithelial cells such as cell fusion between EBV-carrying B lymphocytes and epithelial cells.^{4,23} Further virological studies are recommended to elucidate the biological role of EBV in various types of gastric carcinoma.

EBV-associated gastric carcinomas are characterized by male predominance, although the etiological

significance of sex is unknown even in gastric carcinoma not related to EBV. EBV-related carcinomas were observed in all sites and all histological types, with low prevalence among carcinomas occurring in the antrum, among well-differentiated adenocarcinomas, and among nonsolid type, poorly differentiated adenocarcinomas (mainly of the scirrhous type), signet cell carcinomas, and mucinous carcinomas. Although the number was small, carcinoma in the gastric stump showed the highest prevalence of EBV involvement by site. These clinicopathological characteristic findings of EBV-related gastric carcinoma were statistically significant in multivariate combined analyses as well as in simple analyses for sex, site of tumor, and histological type, and they suggest a different etiological pathway of carcinogenesis from that for non-EBV-related cancers.

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